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THE HUMAN LYMPHOCYTE FUNCTION-ASSOCIATED (HLFA) ANTIGEN AND A RELATED MACROPHAGE DIFFERENTIATION ANTIGEN (HMac-1): FUNCTIONAL EFFECTS OF SUBUNIT-SPECIFIC MONOCLONAL ANTIBODIES¹

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The structural and functional relations between the α - and β -subunits of the human lymphocyte function-associated antigen (HLFA) and the human Mac-1 antigen (HMac-1) have been analyzed with the use of five monoclonal antibodies that react with these proteins. The specificities of these antibodies were examined by immunoprecipitation of proteins from ¹²⁵I-labeled cells and purified HLFA and HMac-1 antigens. Three antibodies reacted with the Mr 95,000 common β -subunit of the proteins, and also co-precipitated the Mr 175,000 HLFA α -subunit, the Mr 165,000 HMac-1 α -subunit, and a third polypeptide α -subunit of Mr 150,000. The other antibodies were specific to noncross-reactive epitopes present on the α -subunits of HLFA or HMac-1. These specificities were confirmed in sequential immunoprecipitation studies. Peptide mapping showed that the β -subunits of HLFA and HMac-1 were identical, whereas the two α -subunits differed considerably. The HLFA α -subunit-specific monoclonal antibody inhibited phytohemagglutinin stimulation, the mixed lymphocytes reaction, cytolytic T lymphocyte-mediated killing, and tetanus toxoid stimulation, but did not affect natural killer cell-mediated killing or complement receptor type 3 function. The HMac-1 α -subunit-specific monoclonal antibody inhibited complement receptor type 3 function but had no effect on T cell or natural killer cell functions. Three monoclonal antibodies to the β -subunit inhibited all functions tested, including T cell, natural killer cell, and complement receptor type 3 activities. The results suggest that the functions of the HLFA and HMac-1 molecules may be determined by the α -subunit, and that the common β -subunit also bears functionally important epitopes.

The human lymphocyte function-associated (HLFA)² antigen is a molecule involved in cytotoxic T lymphocyte (CTL) and natural killer (NK) cell-mediated lysis of target

cells, and is composed of two noncovalently associated polypeptides of Mr 175,000 (α) and 95,000 (β) (1-6). This lymphocyte antigen is equivalent to the previously reported murine LFA-1 antigen which has also been shown to participate in a variety of T cell functions (7-10). HLFA is expressed on T and B lymphocytes as well as thymocytes, granulocytes, and a subpopulation of bone marrow cells. This broad expression of the HLFA antigen and its involvement in NK cell as well as CTL function suggests that it is not an antigen receptor, and there is evidence that the HLFA antigen and its murine homolog may serve as adhesion molecules in the interaction of cells involved in immune responses (2, 7, 10-12).

Both the human and murine LFA molecules show remarkable similarity to a well-characterized murine macrophage differentiation antigen, Mac-1, which contains two subunits of Mr 165,000 (α) and 95,000 (β) (13). A monoclonal antibody (MAb) against murine Mac-1 cross-reacts with human monocytes (14), and has been shown to inhibit binding of C3bi-coated erythrocytes to murine and human macrophages (10, 15). These results, and the results of Wright et al. (16), indicate that the Mac-1 antigen is closely associated with or represents the type three complement receptor (CR₃). Human Mac-1 (HMac-1) appears to be identical in tissue distribution and structure to the OKM-1 and Mo 1 human monocyte antigens (17-19).

Structural studies on murine LFA-1 and Mac-1 antigens have shown that the β -subunits are very similar, if not identical, whereas the α -subunits are considerably different (20-23). Analogous findings have been obtained recently in studies of the equivalent human molecules (6). It was suggested that epitopes involved in T cell function and macrophage complement receptor activity were localized to the α -subunits (23).

In the present study, we used four monoclonal antibodies that react with HLFA, and another that reacts with HMac-1. The specificities of the antibodies were examined by immunoprecipitation of purified HLFA and HMac-1 antigens and sequential immunoprecipitation. Three of the antibodies were specific for the β -subunit; one, the HLFA α -subunit; and one, the HMac-1 α -subunit. Characterization of the antigen showed that the α -subunits of HLFA and HMac-1 as well as a third polypeptide of Mr 150,000 share an identical β -subunits, and that the α -subunits of HLFA and HMac-1 differ. These findings are in substantial agreement with those published recently by Sanchez-Madrid et al. (6). Studies of the functional effects of the antibodies showed that both α -sub-

Received for publication September 4, 1984.

Accepted for publication January 16, 1985.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by grants from the National Institutes of Health (PO1 AI 19373-02), the Office of Naval Research (N00014-82-K-0221), and the Johnson and Johnson Focused Giving Program.

² Abbreviations used in this paper: CR₃, type three complement receptor; CTL, cytotoxic T lymphocytes; DEA, diethylamine-HCl, pH 11.5; HLFA, human lymphocyte function-associated antigen; HMac-1, human Mac-1 antigen; MAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells.

unit-specific and β -subunit-specific MAb inhibit leukocyte function. Moreover, whereas MAb against the HLFA and HMac-1 α -subunits inhibit only the corresponding function (T cell function and CR₃ activity, respectively), MAb against the β -subunit inhibit both. Thus, functional sites have been mapped to epitopes on both the α - and β -subunits.

MATERIALS AND METHODS

Cells. Human peripheral blood mononuclear cells (PBMC) were isolated from normal adults by Ficoll-Hypaque density centrifugation (24). Monocytes were prepared by adherence to plastic petri dishes or by centrifugation of PBMC on Percoll density gradients (25). Cell lines (HSB-2, T lymphoma; JR, B-lymphoblastoid; and U-937, monocyte) were maintained in RPMI 1640 (Biofluids, Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) 2 mM glutamine, and 25 mM HEPES buffer (complete medium).

Monoclonal antibodies (MAb). The production of hybridomas secreting two of the anti-HLFA MAb (MHM 0.23 and MHM 0.24) has been reported (2). Hybridomas secreting additional MAb used in these studies were produced as described (26). In brief, fusion of P3X653-Ag8 myeloma cells with spleen cells from mice immunized with human splenic or peripheral blood-adherent cells was carried out by using 50% (v/v) polyethylene glycol. Supernatant fluids from the resulting hybridomas were screened by immunoperoxidase analysis of antibody binding to cryostat sections (8 μ m) of frozen human tonsils (27). Selected hybridomas were cloned and subcloned as described (26).

Immunoprecipitation. Cells (5×10^7 to 1×10^8) in 0.5 ml of phosphate buffered-saline (PBS) were vectorially iodinated by using lactoperoxidase (Sigma Chemical Co., St. Louis, MO) and H₂O₂ as described (28). The cells were solubilized with lysis buffer (1 ml per 5×10^7 cells) containing 10 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40 (NP-40), 5 mM EDTA, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.14 M NaCl. After 30 min on ice, the detergent-insoluble material was removed by centrifugation at $100,000 \times G$ for 30 min. The supernatant was precleared by incubating 20 min with 0.1 volume of a 10% (w/v) solution of fixed *Staphylococcus aureus*, Cowan I strain, in lysis buffer containing 0.1% (w/v) bovine serum albumin. After centrifugation at $10,000 \times G$ for 5 min, specific membrane proteins were precipitated in two steps. Monoclonal antibodies (0.2 ml of culture supernatant or 0.1 ml of ascites diluted 1/100 in PBS) were incubated with 0.1 ml of precleared extract for 1 hr on ice. Ten micrograms of affinity-purified rabbit anti-mouse IgG (The Jackson Laboratory, Bar Harbor, ME) were added, followed by a 1-hr incubation on ice. Fifty microliters of 10% (w/v) Sac in lysis buffer were added, and after 20 min on ice, the samples were centrifuged at $1500 \times G$ for 10 min. The pellets were washed twice with 20 mM Tris-HCl buffer, pH 7.6, containing 2.5 M KCl, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, and once with 20 mM Tris-HCl buffer, pH 7.6, containing 0.5% NP-40. For one-dimensional sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE), antigens were eluted by boiling for 3 min in Laemmli SDS-PAGE buffer (29). For two-dimensional PAGE, antigens were eluted by incubating for several hours at 37°C in O'Farrell lysis buffer (30). One- and two-dimensional PAGE and autoradiography were performed as described (29, 30).

Purification and iodination of HLFA and Mac-1. Monoclonal antibodies were purified from ascites fluid by (NH₄)₂SO₄ fractionation and ion-exchange chromatography (31). Purified immunoglobulin (Ig) was coupled to CNBr-activated Sepharose beads at a ratio of 2 to 4 mg/ml as described (32). Columns (1.5 \times 7-cm) containing 20 to 40 mg of coupled MAb were washed with 50 ml of DEA elution buffer (50 mM diethylamine-HCl (DEA), pH 11.5, 0.5 NP-40) and were equilibrated with 10 mM Tris-HCl buffer, pH 7.8, containing 0.5% NP-40.

Fresh human spleen (30 to 50 g) was obtained from Dr. J. Burdick, Transplantation Unit, Johns Hopkins Hospital. The spleen was cut into small pieces and was homogenized at 0°C in 200 ml of homogenization buffer (10 mM Tris-HCl, pH 7.8, 5 mM iodoacetamide, 5 mM MgCl₂, 1 mM PMSF, 1 U/ml aprotinin). After centrifugation at $400 \times G$ for 10 min, the pellets were homogenized and were centrifuged as described above. The supernatants were pooled and were centrifuged at $50,000 \times G$ for 30 min to pellet the crude membranes, which were then extracted for 1 hr on ice in 200 ml homogenization buffer containing 2% NP-40. After centrifugation at $100,000 \times G$ for 1 hr, the supernatant was passed through the HLFA or HMac-1 MAb affinity column. The columns were washed extensively with 10 mM Tris-HCl buffer, pH 7.8, containing 0.5 M NaCl

and 0.5% NP-40. The antigens were then eluted by using 2 column volumes of pH 11.5 DEA elution buffer or 50 mM glycine, pH 2.5, containing 0.5% NP-40. Eluted fractions were neutralized immediately by using 2 M Tris HCl, pH 7.5. Purified antigens were iodinated by using chloramine-T as described (33).

Phytohemagglutinin (PHA) stimulation. PHA stimulation was carried out in 96-well, round-bottomed microculture plates (Linbro Chemical Co., New Haven, CT). PBMC (1 to 2×10^5) in 50 μ l complete medium were mixed with an equal volume of MAb (immune ascites fluid) diluted 10^{-2} to 10^{-6} with complete medium. After 20 to 30 min at 25°C, 50 μ l of complete medium containing PHA were added for a final lectin concentration of 0.25 to 1 μ g/ml. The cells were incubated for 3 days at 37°C in a CO₂ incubator. Proliferation was measured by uptake of [³H]thymidine ([³H]TdR; 1 mCi/well) (Amersham, Arlington Heights, IL) during the final 18 hr of culture. Cells were harvested on glass fiber filters by using an automated harvester (PHD; Cambridge Technology, Cambridge, MA).

Mixed lymphocyte reaction (MLR). PBMC depleted of adherent cells by incubating 1 hr at 37°C in plastic petri dishes were incubated for 20 min at room temperature with monoclonal antibodies at various dilutions in complete medium. These cells were then cocultured in round-bottomed microculture plates at 1×10^6 /ml in complete media with allogeneic adherent cells (7×10^5 /ml) that had been treated with mitomycin C (100 μ g/ml) for 1 hr at 37°C. After 5 days at 37°C, 1 μ Ci of [³H]TdR was added to the wells. Twenty-four hours later, the cells were harvested and were assayed for isotope incorporation as described above.

Tetanus toxoid-induced proliferation. Assays were carried out in round-bottomed microculture plates. PBMC at 4×10^6 /ml were incubated for 30 min at 25°C with an equal volume (50 μ l) of MAb diluted 10^{-2} to 10^{-6} with complete medium. Fifty microliters of complete medium containing 0.75 or 1.5 μ g of tetanus toxoid (Wyeth Laboratories, Marietta, PA) were then added. After 4 days at 37°C, 1 μ Ci of [³H]TdR was added to the wells. Cells were harvested and were assayed for isotope incorporation 24 hr later as described above.

CTL-mediated lympholysis. Stimulator cells (PBMC) in complete medium were treated with mitomycin C (100 μ g/ml) for 1 hr at 37°C at a density of 40×10^6 cells/ml. After four washes with complete media, the stimulator cells were cocultured with an equal number (80×10^6) of allogeneic responder PBMC in 40 ml of complete media. After 7 days, the cells were harvested, were washed twice with complete media, and were adjusted to a density of 6×10^6 /ml in complete media for use as effector CTL. Untreated stimulator cells were cultured separately as a source of target cells. These cells were washed once with RPMI 1640 and were incubated for 1 hr at 37°C in complete media containing 1 mCi of Na⁵¹CrO₄ (Amersham). The cells were then washed three times and were suspended at 2×10^5 /ml in complete medium. Effector CTL (3×10^5) in 50 μ l of complete medium were incubated with an equal volume of diluted antibody or medium for 30 min at room temperature in round-bottomed microculture plates, before adding 50 μ l of the labeled target cells (effector to target ratio = 30). The cells were incubated for 5 to 6 hr at 37°C, the plates were centrifuged at $400 \times G$ for 5 min, and 80 μ l of supernatant were removed to assay isotope release. Controls for spontaneous release (target cells plus medium) and total release (target cells lysed in 100 μ l of 5% Triton X-100) were dispensed in four wells while experimental assays were carried out in duplicate wells. Spontaneous release did not exceed 20%. Percent specific lysis was calculated according to the standard formula (34).

NK cell-mediated lympholysis. NK cell activity was generated by incubating PBMC at 3×10^6 /ml in complete medium for 4 days in a T-75 culture flask (Costar, Cambridge, MA). Cells were then washed and were adjusted to 6×10^6 /ml in complete medium. K-562 erythroleukemia cells, labeled with Na⁵¹CrO₄ as described above, were used as target cells. The NK cell assay was carried out in the absence or presence of monoclonal antibodies as described above for CTL.

Tryptic peptide mapping studies. Tryptic peptide analysis was performed as described (35). In brief, iodinated HLFA and MAC-1 were immunoprecipitated and the subunits were separated by SDS-PAGE. The bands were cut from dried gels, were rehydrated in 0.5 ml of 50 mM NH₄HCO₃, and were washed with 1 ml of 50 mM NH₄HCO₃. The gel slices were then ground into fine pieces in 0.5 ml of 50 mM NH₄HCO₃. Digestion with TPCK-trypsin was carried out for 44 hr at 37°C with 30- and 20- μ l additions of a 1 mg/ml trypsin solution at 0 and 24 hr, respectively. After lyophilization, peptides were taken up in electrophoresis buffer and were analyzed on thin-layer cellulose plates (20 \times 20-cm; EM Laboratories, Inc., Elmsford, NY) by high voltage electrophoresis in the first dimension, followed by chromatography in the second dimension essentially as described (35). Single-dimensional peptide maps after digestion with trypsin, papain, or formic acid were generated by using the method of Cleveland et al. (36).

Type three complement receptor (CR₃) assay. Human C3-inacti-

vator and sheep erythrocytes sensitized with guinea pig IgM and human complement components (EAClgp4.2,3hu) were purchased from Cordis Laboratories, Miami, FL. EAClgp4.2,3hu (5×10^6) were incubated with 250 U of C3-inactivator in 1 ml of gelatin-Veronal buffer for 1 hr at 37°C. The cells were then centrifuged at $500 \times g$ for 10 min and were resuspended in 1 ml of Hanks' balanced salt solution (HBSS).

Monocytes (2×10^6) in 2 ml of complete media were incubated for 2 hr at 37°C in Linbro multiwell plates. Nonadherent cells were removed by washing with HBSS, and 1 ml of a 1/100 dilution of MAb (ascites fluid) in HBSS was added to the wells. The adherent cells were incubated for 30 min at room temperature before adding 50 μ l of the C3-inactivator-treated EAClgp4.2,3hu suspension. The incubation was continued for 1 hr at room temperature and then the unbound erythrocytes were gently washed away with HBSS. The wells were examined by using an inverted microscope. Monocytes rosetting ≥ 3 EAClgp4.2,3hu were scored as positive.

RESULTS

Specificities of HLFA and HMac-1 antibodies. A large number of MAb was obtained after fusion of myeloma cells with spleen cells of BALB/c mice immunized with human monocytes, macrophages, or low density adherent cells. These MAb were tested for their effect on in vitro functional assays, and two MAb (H52, H5B9) were found which inhibited a wide range of T cell functions (see below). These two antibodies precipitated polypeptides from the T cell line HSB-2 with Mr values (175,000 and 93,000) identical to those of polypeptides precipitated by two previously reported anti-HLFA MAb, MHM.23 and MHM.24 (1) (Fig. 1). Immunohistologic studies showed that the staining patterns of H52 and H5B9 MAb on human tonsil and spleen sections were identical to that of MHM.23 (not shown). (The epitope recognized by MHM.24 was apparently sensitive to the fixatives used in preparing the sections because it consistently failed to react with the fixed tissues.)

Another MAb prepared in this study, H5A4, also precipitated a polypeptide of Mr 93,000 as well as a polypeptide of Mr 165,000 when tested against extracts of human monocytes under either reducing or nonreducing

conditions (Fig. 2). Immunohistologic screening showed that this MAb stained only cells with macrophage morphology. This antibody failed to precipitate any antigens from T cells or B cells (not shown). H5A4 thus appeared to recognize the human equivalent (HMac-1) of the previously reported mouse macrophage antigen Mac-1 (13).

As shown in Figure 2, polypeptides of Mr 175,000 and 93,000 were precipitated from monocytes by the four HLFA MAb. In addition, three of the MAb (H52, H5B9, MHM.23) also precipitated a third polypeptide of Mr 150,000 (p150) from monocytes. The p150 polypeptide was consistently precipitated from monocytes or macrophages by these three MAb, but not from other cell types including the HSB-2 cell line, as shown above. The amount of the p150 polypeptide present in the precipitates was not related to the length of the assay nor to the presence of protease inhibitors. Precipitation conditions were identical for all cell types used, suggesting that the p150 polypeptide does not represent a breakdown product of the HLFA α -chain (p175) or the HMac-1 α -chain.

Two-dimensional isoelectric focusing and SDS-PAGE studies. The subunits of the HLFA and HMac-1 antigens were compared by two-dimensional isoelectric focusing and SDS-PAGE. The two-dimensional patterns of polypeptides precipitated by the four anti-HLFA were identical in having only two broad bands corresponding to the α -(Mr 175,000) and β -(Mr 93,000) subunits (Fig. 3). Both subunits focused at pI 5.1 to 5.3 with multiple components, possibly reflecting sialic acid charge heterogeneity typical of many glycoproteins. The anti-HMac-1 MAb (H5A4) did not precipitate any antigen from HSB-2 cells (not shown).

The two-dimensional patterns of immunoprecipitates from monocytes were quite unlike those from the HSB-2 T cell line (Fig. 4). The MHM.24 and H5A4 MAb precipitated only two bands corresponding to the α -subunit and β -subunit of HLFA and HMac-1, respectively (Fig. 4a). There was no evidence of any additional subunits in either the MHM.24 or H5A4 immunoprecipitates even after prolonged exposure of the gels. Three of the HLFA MAb (H52, H5B9, MHM.23) precipitated four distinct

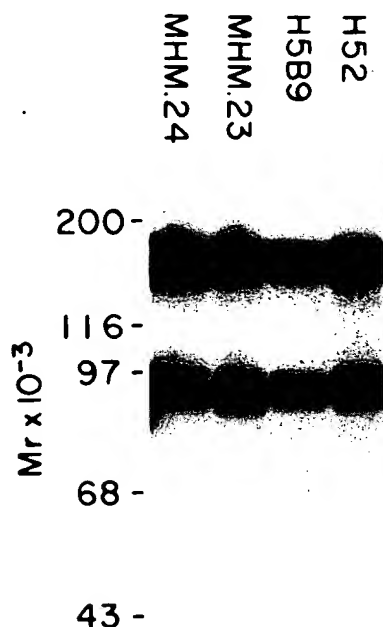


Figure 1. Immunoprecipitation of HLFA from the HSB-2 cell line. HSB-2 cells were labeled with 125 I and were extracted with 0.5% NP-40. Antigens were immunoprecipitated with 50 μ l MAb (1/100 diluted ascites fluid). Reduced precipitates were subjected to 7.5% SDS-PAGE and autoradiography.

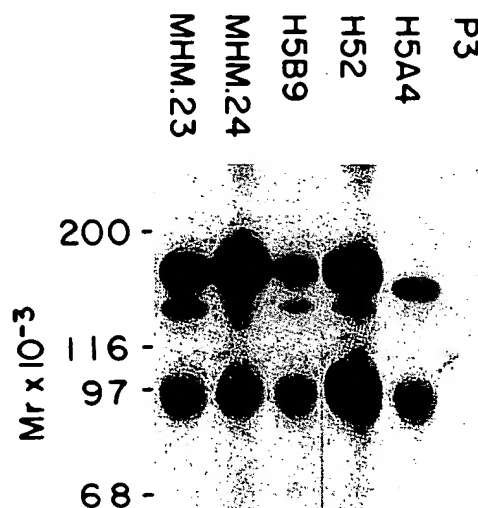


Figure 2. Immunoprecipitation of HLFA and HMac-1 antigens from monocytes. Monocytes were isolated from peripheral blood on a Percoll gradient (25) and were labeled with 125 I. After extraction with 0.5% NP-40, antigens were immunoprecipitated with MAb or supernatant from the P3x653-Ag8 myeloma line (P3), and were analyzed under reducing conditions as described for Figure 1.

Figure 3. Two-dimensional IEF-PAGE analysis of HLFA antigens precipitated from HSB-2 cells. HSB-2 cells were surface-labeled with ^{125}I and were extracted with 0.5% NP-40. Antigens were immunoprecipitated with MAb as described for Figure 1. Two-dimensional electrophoresis was performed as described by O'Farrell (30). Antigens were visualized by autoradiography. $L\alpha$ = HLFA α -subunit; β = β -subunit.

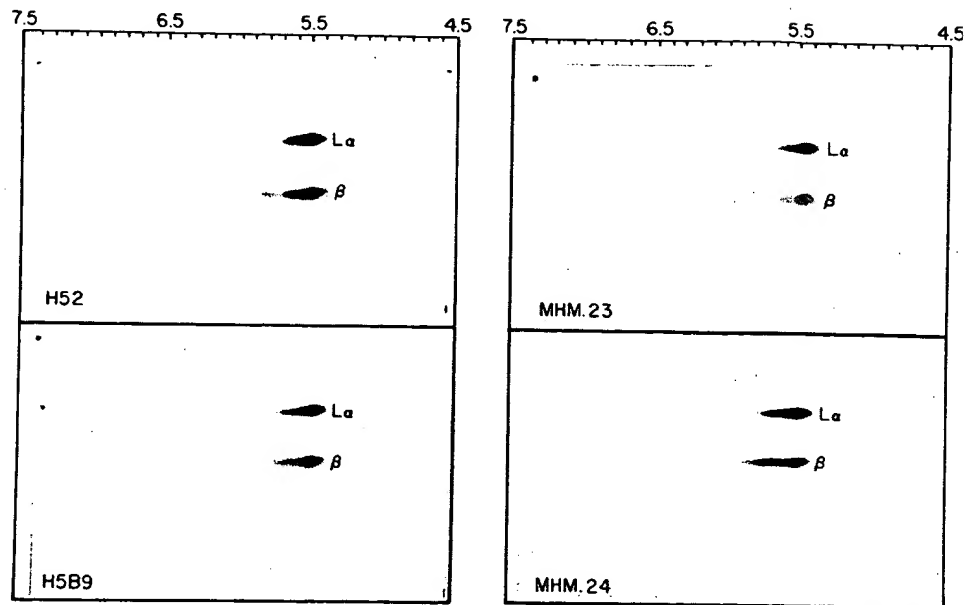
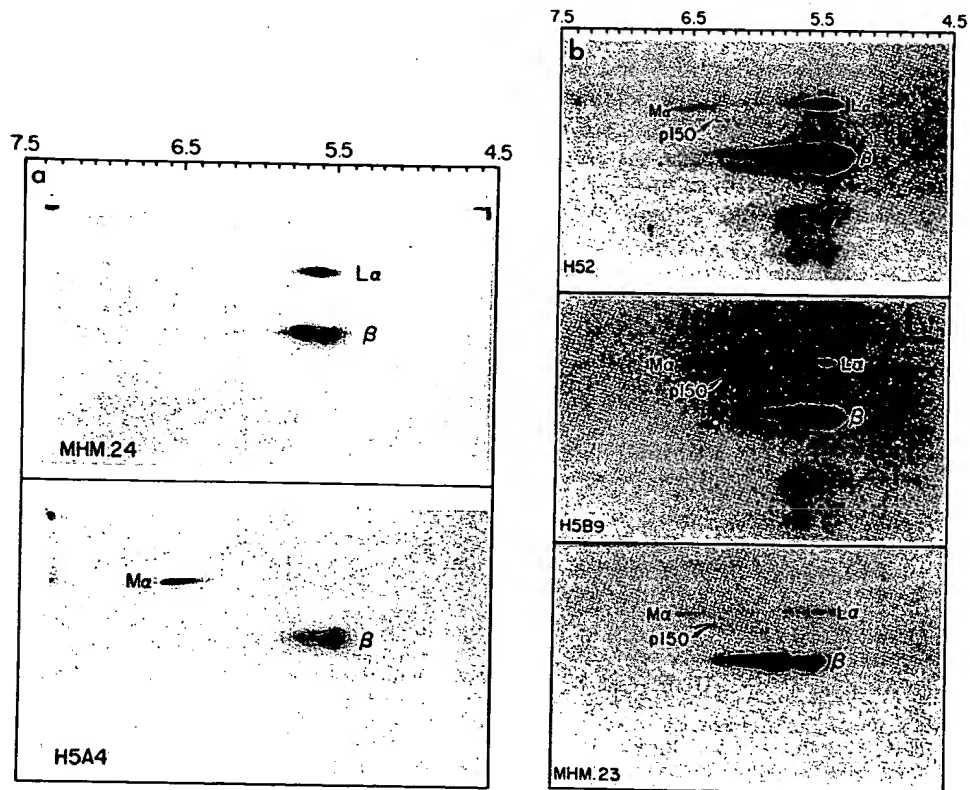


Figure 4. Two-dimensional IEF-PAGE analysis of HLFA and HMac-1 antigens precipitated from monocytes. Monocytes were prepared from peripheral blood on Percoll gradients (25) and were labeled with ^{125}I . After extraction with 0.5% NP-40 lysis buffer, antigens were immunoprecipitated and were analyzed by two-dimensional electrophoresis as described for Figure 3. $L\alpha$ = HLFA α -subunit; $M\alpha$ = HMac-1 α -subunit; p150 = Mr 150,000 subunit; β = β -subunit.



bands, two of which correspond to the α - and β -subunits of HLFA. In addition, however, were minor bands at Mr 165,000 and Mr 150,000 daltons. The Mr 165,000 chain was identical in Mr and pI (6.6) to the HMac-1 α -subunit precipitated by H5A4 (Fig. 4b). The Mr 150,000 chain was slightly more acidic, focusing at pI 6.4. Mixing experiments showed that the Mr 165,000 and β -subunit precipitated by H5A4 co-migrated with the corresponding polypeptides precipitated by H52. Similarly, the Mr 175,000 and β -subunit precipitated by MHM.24 co-migrated with the corresponding subunits of H52 immunoprecipitates (data not shown). The results suggested that the H52, H5B9, MHM.23 MAb were specific for an epitope on a β -subunit associated with multiple α -chains, or that these MAb reacted with an epitope shared by the three α -subunits. These results further suggested that the

MHM.24 and H5A4 MAb were specific for determinants on the HLFA and HMac-1 α -subunits, respectively.

Studies using affinity-purified HLFA and HMac-1. The subunit specificities of the MAb were further examined with HLFA and HMac-1 purified from human spleen by MAb immunoaffinity chromatography. The purified HLFA and HMac-1 antigens were iodinated and were used in immunoprecipitation studies.

The protein eluted from the MHM.23 affinity column at pH 11.5 was similar to antigen immunoprecipitated from monocytes by this antibody, including polypeptides of Mr 175,000, 165,000, 150,000, and 93,000 (Fig. 5a). Some low m.w. contaminating proteins were also observed. H52 and H5B9 MAb both precipitated only the β -subunit, suggesting that the subunits were separated by the high pH elution buffer and that the epitope recognized

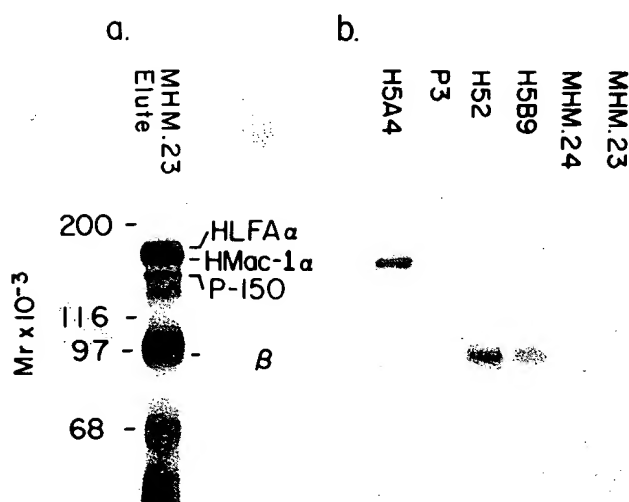


Figure 5. Immunoprecipitation of antigens purified by MHM.23 immunoaffinity. Proteins purified as described in *Materials and Methods* were labeled with ^{125}I by using chloramine-T (33), and were immunoprecipitated with the indicated MAb or the supernatant from the P3 myeloma line. *a*, ^{125}I -labeled purified protein after 7.5% SDS-PAGE and autoradiography showing HLFA α , HMac-1 α , p150, and β . *b*, Immunoprecipitates from purified proteins shown in *a*. The precipitates were analyzed as described for Figure 1.

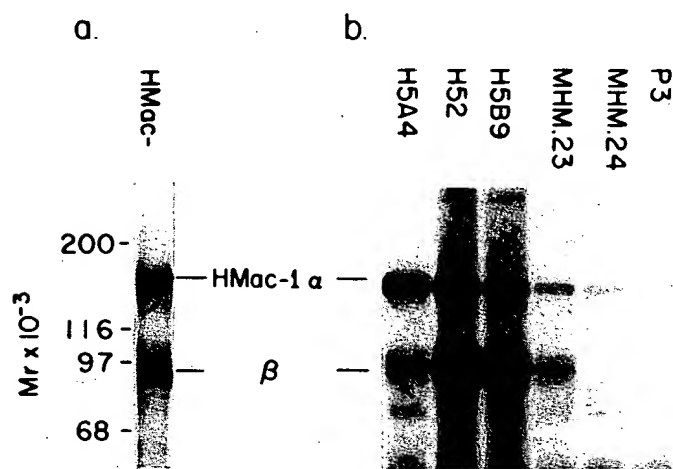


Figure 6. Immunoprecipitation of antigens purified by H5A4-immunoaffinity chromatography. Proteins were purified as described in *Materials and Methods*. After labeling with ^{125}I , antigens were immunoprecipitated with the indicated MAb or P3 myeloma line supernatant. *a*, ^{125}I -labeled purified HMac-1 after 7.5% SDS-PAGE and autoradiography showing HMac-1 α and the β -subunit. *b*, Immunoprecipitates from purified HMac-1 were subjected to 7.5% SDS-PAGE and autoradiography.

by these antibodies is on the β -subunit. Separation of the α - and β -subunits by pH 11.5 has been reported (6). H5A4 precipitated the HMac-1 α -subunit, Mr 165,000, but not the β -subunit, indicating that this MAb is specific for HMac-1 α . Precipitation of the HMac-1 α -subunit from antigens purified on the MHM.23 affinity column confirmed the presence of HMac-1 α subunits in the MHM.23-purified protein. The MHM.23 and MHM.24 MAb did not precipitate any polypeptides from the labeled, purified antigens, suggesting that the epitopes identified by these MAb are sensitive to the elution conditions (pH 11.5) or that the conformation of the epitopes require association of the α - and β -subunits.

The results of immunoprecipitation studies using purified HMac-1 are shown in Figure 6. This protein was eluted from the H5A4 column at pH 2.5 more efficiently than at pH 11.5. The purified antigen eluted at pH 2.5 consisted of the α - and β -subunits of HMac-1 (Fig. 6a).

Four of the MAb (H5A4, H52, H5B9, and MHM.23) each precipitated both the α - and β -subunits (Fig. 6b), indicating that only limited dissociation of the subunits had occurred. In three separate experiments, the MHM.24 precipitate contained a very faint band migrating at the HMac-1 α position, but no band at the β position (Fig. 6b). However, no HMac-1 α -chain was observed in MHM.24 immunoprecipitates from labeled HMac-1-positive cells. These results indicate that MHM.24 might react weakly with isolated HMac-1 α but it clearly does not react with intact HMac-1. Precipitation of the HMac-1 antigen by the MHM.23, H52, and H5B9 MAb indicates that the β -subunit associated with the HMac-1 α -chain is identical to or has epitopes shared by the β -subunit associated with HLFA α -chain.

Sequential immunoprecipitation studies. Monocyte cell surface proteins were iodinated and were extracted with detergent as described in *Materials and Methods*. Small aliquots of extract were depleted of labeled antigens by two cycles of immunoprecipitation with an excess of either anti-HMac-1- α (H5A4), anti-HLFA- α (MHM.24), or anti- β (H52) MAb. The depleted extracts were then used for immunoprecipitations with each of the three MAb (Fig. 7).

Virtually all of the antigens recognized by H52 (anti- β) were removed by pre-clearing with this antibody. MHM.24 (anti-HLFA- α) precipitated only the HLFA α -chain from the extract cleared with H52. H5A4 (anti-HMac-1- α) precipitated HMac-1 α -chain and very little β -subunit.

Pre-clearing with MHM.24 (anti-HLFA- α) resulted in the complete removal of all MHM.24-reactive antigens. H52 (anti- β) precipitated β -subunits from the extract cleared with MHM.24, along with weak bands at the HMac-1- α and Mr 150,000 positions. The HMac-1 antigens were not depleted by MHM.24 (anti-HLFA α), as shown by the H5A4 (anti-HMac-1- α) precipitate from the MHM.24-cleared extract.

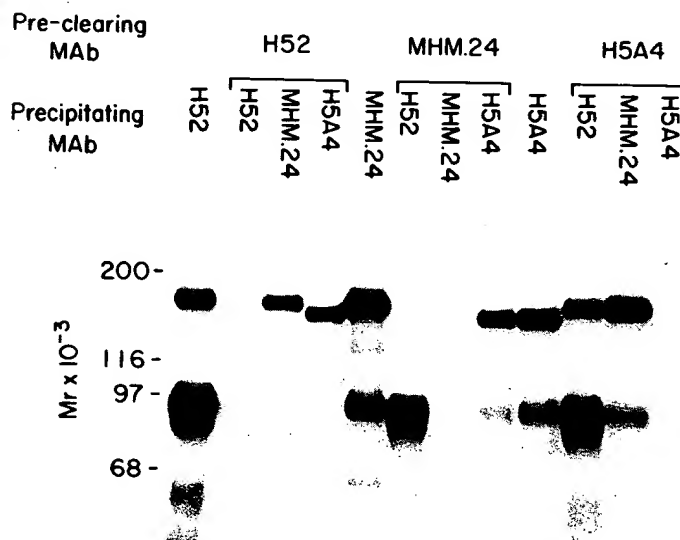


Figure 7. Sequential immunoprecipitation by using anti- β (H52), anti-HLFA- α (MHM.24), and anti-HMac-1- α (H5A4) MAb. Extracts of 5×10^6 surface-iodinated monocytes were subjected to two cycles of immunoprecipitation with 50 μl of diluted (1/100) H52, MHM.24, or H5A4 ascites fluid as described in *Materials and Methods*. The supernatants from the final immunoprecipitation were divided into three equal parts and were immunoprecipitated with each of the three MAb as indicated. Precipitates were analyzed by SDS-PAGE and autoradiography as described for Figure 1.

Pre-clearing with H5A4 (anti-HMac-1- α) completely depleted HMac-1 antigens from the extract but did not affect HLFA antigens, as indicated by the H52 and MHM.24 precipitates from H5A4-cleared extract (Fig. 7).

The results of these studies and the above two-dimensional and immunoaffinity chromatography studies showed that the H52, H5B9, and MHM.23 MAb were specific for a shared β -subunit, whereas the MHM.24 and H5A4 MAb were specific for the HLFA α -subunit and HMac-1 subunit, respectively. In other studies (not shown), the H5B9 and MHM.23 MAb precipitated only β -subunits from MHM.24-cleared extract. The results of the sequential immunoprecipitation experiments also showed that non-associated α - and β -subunits were present in the monocyte membrane extracts used for immunoprecipitations.

Tryptic peptide mapping. All of the above data suggested strongly that HMac-1 and HLFA antigens consisted of an identical β -subunit associated with different α -subunits. The extent of the homology between HLFA- β and HMac-1- β was investigated by peptide mapping analysis. The antigens were immunoprecipitated from iodinated monocytes, and the α - and β -subunits were separated by SDS-PAGE. After excising the radioiodinated bands from the gels, tryptic peptides were prepared and were analyzed on thin-layer cellulose plates by electrophoresis and chromatography. The tryptic peptide maps of the β -chains precipitated by the H52 and H5A4 were identical (Fig. 8). In contrast, although the map of the HLFA-1 α -subunit showed eight major spots, the HMac-1 α -subunit consistently showed only one major spot, and three faint spots which did not correspond overlap with

those of HLFA-1- α . Similar results were obtained when peptides were generated with papain or formic acid and were analyzed by one-dimensional SDS-PAGE as described by Cleveland et al. (36) (data not shown).

Effects of MAb on lymphocyte function. The availability of MAb specific for the common β -subunit and distinct α -subunits of the HLFA and HMac-1 antigens made it possible to determine whether the function of these antigens could be mapped to a particular subunit. These experiments were performed with MAb in the form of heat-inactivated ascites fluid (MAb content 1 to 5 mg/ml) at a final dilution of 1/100 in complete medium. Indirect trace-binding assays showed that this concentration would give saturation binding of all MAb used in the functional assays (data not shown). A previous study indicated that anti-HLFA MAb inhibit lymphocyte function at the responder cell level (2); therefore, in all experiments, responder cells were preincubated 20 to 30 min with the various MAb before adding antigen, mitogen, or stimulator cells. All MAb used were of the same isotype (IgG1, κ).

Mitogenic stimulation of T cells by a suboptimal dose of PHA (0.3 μ g/ml) was markedly inhibited (>80%) by the anti-HLFA- α MAb (MHM.24) and the three anti- β MAb (H52, H5B9, MHM.23) (Table I). The same degree of inhibition was obtained with MAb concentrations as low as 0.1 μ g/ml (not shown). When a 10-fold greater concentration of PHA was used (3 μ g/ml), the three anti- β MAb inhibited proliferation as much as 50%, whereas the anti-HLFA- α MAb (MHM.24) had no effect (data not shown). The anti-HMac-1- α MAb (H5A4) did not significantly inhibit PHA stimulation (Table I).

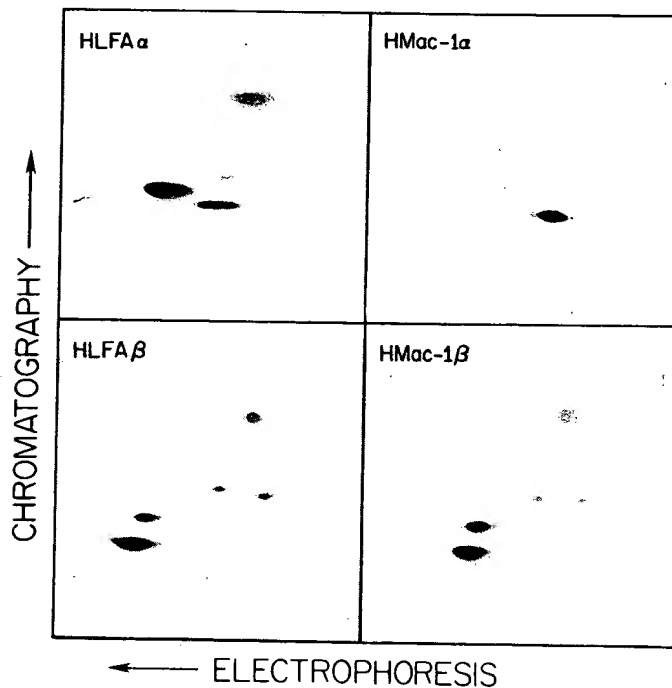


Figure 8. Tryptic peptide maps of iodinated HLFA and HMac-1 subunits. HLFA and HMac-1 antigens were immunoprecipitated from surface-iodinated monocytes with MHM.24 and H5A4 MAb, respectively. After separation by 7.5% SDS-PAGE, the subunits were excised from the gels, were digested with TPCK-trypsin, and analysis by two-dimensional electrophoresis and chromatography was carried out as described (35). The HLFA α - and β -subunits contained approximately 10,000 cpm each and were autoradiographed for 10 days. The HMac-1 α - and β -subunits contained approximately 5000 cpm each and were autoradiographed for 14 days.

TABLE I
Effect of MAb on lymphocyte function^a

MAb	Isotype	Specificity	% Inhibition of Function					
			PHA ^b	MLR ^c	tetanus toxoid ^d	CTL ^e	NK ^f	CR ₃ ^g
H52	IgG1, κ	anti- β	90	100	75	97	76	75
H5B9	IgG1, κ	anti- β	92	99	67	99	75	70
MHM.23	IgG1, κ	anti- β	88	98	55	95	55	59
MHM.24	IgG1, κ	anti-HLFA- α	79	98	32	100	0	19
H5A4	IgG1, κ	anti-HMac-1- α	10	15	12	0	0	70
H53	IgG1, κ	anti-HLA-DR	12	100	98	0	0	17

^a Inhibition of leukocyte function by HLFA and HMac-1 subunit-specific MAb. Immune ascites fluid diluted 1/100 in culture medium was used as a source of MAb in all experiments.

^b PBMC were incubated with an equal volume of MAb for 20 min at room temperature before adding PHA to a final concentration of 0.3 μ g/ml. Proliferation was assayed after 3 days of culture. Incorporation of [³H]TdR in the absence of MAb was 17,550 cpm.

^c Responder PBMC were incubated with an equal volume of MAb for 20 min at room temperature before adding stimulator cells as described in *Materials and Methods*. The MLR was assayed for incorporation of [³H]TdR on day 6 of culture. Incorporation in the absence of MAb was 21,120 cpm.

^d PBMC were incubated with an equal volume of MAb for 30 min at room temperature before adding tetanus toxoid to a final concentration of 5 μ g/ml. After 4 days of culture, cells were assayed for incorporation of [³H]TdR. Incorporation in the absence of MAb was 13,162 cpm.

^e Primary allogeneic CTL were pretreated with equal volumes of MAb for 30 min at room temperature; ⁵¹Cr-labeled target cells were then added (E:T = 30:1) and the assay was continued for 6 hr. The specific release of ⁵¹Cr was 25% in the absence of MAb.

^f The NK assay was carried out as described for CTL. The E:T ratio was 30:1 and the spontaneous ⁵¹Cr release was 12%. Specific release in the absence of MAb was 32%.

^g Monocytes adherent to 2-ml culture wells were treated for 30 min at room temperature with MAb before adding C3-inactivator-treated sheep EAC1gp4.2.3.hu. After an additional hour at room temperature, unbound erythrocytes were removed by washing and the monocytes were examined for rosetting. EAC1gp4.2.3hu not treated with C3-inactivator did not bind to the monocytes.

The three anti- β MAb completely blocked the antigenic stimulation of T cells in the MLR (Table I). The response was also significantly inhibited (89%) by the anti-HLFA- α MAb (MHM.24). Concentrations of these four MAb as low as 0.1 μ g/ml gave maximal inhibition of the MLR (data not shown). An MAb (H53) recognizing a monomorphic class II MHC determinant also completely inhibited the MLR, in keeping with the role of class II MHC antigens as the primary antigenic stimulus in the MLR (37). The proliferation of T cells in the MLR was only marginally reduced (15%) by the anti-HMac-1- α MAb (H5A4) (Table I).

In addition to their responses to lectin mitogens and allogeneic cell surface antigens, T cells also respond to soluble antigens presented on the surface of class II MHC-matched accessory cells (38). The effects of the MAb on such responses were tested, using tetanus toxoid as soluble antigen. At the optimal dose of antigen (5 μ g/ml), the three anti- β MAb inhibited proliferation by as much as 75% (Table I). These MAb blocked proliferation equally well at sub-saturating concentrations (0.1 μ g/ml). The anti-HLFA- α MAb (MHM.24) also inhibited soluble antigen-driven proliferation, but less effectively (32%). Proliferation was completely inhibited by the anti-HLA-DR MAb (H53). In contrast, the anti-HMac-1- α MAb (H5A4) did not significantly reduce the proliferative response to tetanus toxoid (Table I).

The above studies showed that MAb against the HLFA α -chain and the β -subunit inhibit the induction phase of *in vitro* T cell responses. Previous studies have shown that anti-HLFA MAb also inhibit the effector phase of lymphocyte functions (1). The effects of the MAb on the effector phase of lymphocyte responses were tested in CTL and NK cell-mediated lympholysis assays. The three anti- β MAb and the anti-HLFA- α MAb completely inhibited target cell lysis by allospecific CTL (Table I). In some experiments, complete inhibition could be obtained with these antibodies at concentrations as low as 10 ng/ml (data not shown). The anti-HMac-1- α MAb (H5A4) had no effect on CTL activity.

The three anti- β MAb also significantly inhibited NK cell lysis of target cells (up to 76%) (Table I). In contrast to its effect on CTL activity, the anti-HLFA- α MAb (MHM.24) did not inhibit NK cell function. Similarly, NK cell activity was not affected by the anti-HMac-1- α MAb (H5A4).

MAb against the α -subunit of Mac-1 have been shown to inhibit CR₃ activity (10, 15, 16). As shown in Table I, the anti-HMac-1- α MAb (H5A4) significantly reduced C3bi-coated erythrocyte binding to monocytes, whereas the anti-HLFA- α MAb (MHM.24) inhibited no better than did a control anti-HLA-DR MAb (H53). All three anti- β -subunit MAb (H52, H5B9, MHM.23) markedly inhibited CR₃ activity.

DISCUSSION

The murine LFA-1 and Mac-1 antigens are structurally related cell surface molecules involved in lymphocyte and macrophage functions, respectively (10). The LFA-1 and Mac-1 antigens consist of α -subunits of Mr 175,000 and 165,000, respectively, noncovalently associated with an identical β -subunit of Mr 93,000. We have shown that the human equivalents of these murine antigens (HLFA and HMac-1) consist of subunits with m.w. and structural

organization identical to those of LFA-1 and Mac-1. These results are in agreement with those recently published by Sanchez-Madrid et al. (6). We further demonstrated that MAb against the shared β -subunit of HLFA and HMac-1 inhibit the function of both molecules, whereas MAb against the distinct α -subunits inhibit only the corresponding function.

The structural relationship between HLFA and HMac-1 was studied by using five MAb. Two-dimensional PAGE analysis of immunoprecipitates from monocytes showed that an anti-HMac-1 (H5A4) and an anti-HLFA (MHM.24) MAb precipitated α -subunits of distinct Mr and pI and β -subunits with identical Mr and pI, suggesting that these MAb recognized epitopes on the α -subunits. Three MAb (H52, H5B9, MHM.23) precipitated the β -subunit as well as molecules identified as the HLFA- α -subunit, HMac-1 α -subunit, and an Mr 150,00 polypeptide. Tyrosyl tryptic two-dimensional peptide maps of the β -subunits of HLFA and HMac-1 were identical, whereas the peptide maps of the α -subunits were different. The results were similar for single-dimensional Cleveland maps (36) of peptides generated with papain or formic acid. The data indicated that a common β -subunit was shared by HLFA- α , HMac-1- α , and an Mr 150,000 polypeptide, in agreement with results obtained by other investigators (5, 6, 21). The precipitation of multiple α -subunits and the β -subunits could have been due to recognition of the shared β -subunit or a common α -subunit determinant. Results with affinity-purified antigens and sequential immunoprecipitations showed clearly that the cross-reactive MAb were specific for an epitope or epitopes on the β -subunit, whereas the unique epitopes recognized by HLFA and HMac-1 MAb were found on the α -subunits. A similar subunit organization and epitope segregation exist for class I MHC antigens in which a shared subunit (β_2 -microglobulin) carries a common epitope(s) and the distinct heavy chains bear unique polymorphic determinants (39).

Studies of the subunit structure of HLFA have shown that the antigen is present in an α , β structure (6). However, the sequential immunoprecipitation studies reported here revealed that free, non-associated α - and β -subunits were present in detergent extracts of labeled cells. This was especially true for the HLFA subunits. When all HLFA α -subunits were depleted from extracts of monocytes, the anti- β MAb precipitated a large amount of β -subunit and very little HMac-1 α , whereas the anti-HMac-1 precipitated both the α - and β -subunits of HMac-1. In the reciprocal experiments with HMac-1-depleted extracts, the anti- β and anti-HLFA- α MAb precipitated both the HLFA α - and β -subunits. These results suggested either that the free pool of β -subunits was large compared to the amount of HMac-1 antigens in the extract, or that the anti- β MAb showed preferential binding to free β and HLFA- α -associated β . These findings could be due to the presence of non-associated subunits in cell membranes, dissociation of the subunits by the extraction procedure, or dissociation of the subunits by binding of MAb. Dissociation of the subunits during extraction or analytical manipulation is possible, given the dissociation of HLFA antigens observed during affinity purification procedures. However, the normal presence of non-associated subunits in the cell membrane cannot be ruled out. Studies of subunit structure by cross-linking experiments also

revealed non-cross-linked α - and β -subunits (6), reflecting either the inefficiency of cross-linking or the presence of non-associated subunits.

The results of functional studies emphasize the importance of HLFA and HMac-1 in cellular interactions. The MAb specific for the HLFA α -subunit (MHM.24) inhibited T cell responses to PHA, tetanus toxoid, class II MHC antigens (MLR), and also completely inhibited CTL function. This antibody did not affect NK cell or CR₃ activities. In a previous study, the MHM.24 MAb inhibited NK cell activity when NK cells were generated by using an Epstein Barr virus-transformed β -cell line (2). This probably reflects a contribution by T cells to the lysis observed (40). The MAb (H5A4) against the HMac-1 α -subunit did not affect T cell or NK cell function but blocked CR₃ activity, in agreement with previous reports indicating that Mac-1 is equivalent to CR₃ (10, 15, 16). The three β -subunit-specific MAb (H52, H5B9, MHM.23) markedly inhibited all functions tested, including T cell, NK cell, and CR₃ activities. Inhibition of CTL and NK activity was also reported by Beatty et al. (3) with an antibody, 60.3, that appeared very similar to MHM.23, competing with MHM.23 for antigen binding, and by Krensky et al. (4) with an independently obtained anti-human β -MAb. In contrast is a previous report in which a murine β -subunit-specific MAb failed to inhibit both T cell function and CR₃ activity (23). The failure of the anti- β -MAb to affect function in the previous study could be due to recognition of an inappropriate epitope. It is possible that β -specific MAb are effective inhibitors only if they recognize epitopes involved in maintaining the conformation of the α - β complex. In analogy to HLA-A,B,C antigens, binding of MAb to such β -epitopes could affect the conformation of the α -subunit (41). MAb against the β -subunit could also act by binding to an epitope spatially close to an α -subunit functional site, resulting in steric hindrance. It is very likely that the functional activity of the HLFA and HMac-1 antigens (lymphocyte interactions and CR₃ activity, respectively) is determined by the α -subunits. Thus, in the present study, the HLFA α -specific MAb inhibited only T cell functions, whereas the HMac-1 α -specific MAb inhibited only CR₃ activity. Similar results have been reported for mouse proteins (23). The β -subunit may serve a function common to both HLFA and HMac-1, such as signal transduction. The broad inhibition of function by MAb against the appropriate β -subunit epitope may be explained by the association of a common β -subunit with multiple function-determining α -subunits.

A polypeptide of Mr 150,000 (p150) was also found to be associated with the β -subunit. This molecule, also reported in a similar study (6), appears to be a third α -subunit and was detected on cells that also express HMac-1. The HMac-1-positive lymphocyte subpopulation has been shown to be highly enriched for NK cells (14). NK cell activity was not affected by the anti-HLFA- α or anti-HMac-1- α MAb, but was blocked by MAb against the β -subunit, which is associated with the p150 molecule. Thus, the p150 polypeptide may represent a third α -chain involved in NK cell function. The production of p150-specific MAb will make it possible to test this hypothesis.

It has been suggested that LFA antigens serve as adhesion molecules (2, 7, 10, 11, 12). This suggestion is supported by our observation that, when anti- β or anti-HLFA- α MAb were added to functional assays, the cell

conjugates which usually appeared before effector function did not form (Hildreth, unpublished observation). A similar adhesion function is apparently served by the HMac-1 antigen or a closely associated structure, because we have shown that an MAb against the HMac-1 α -subunit blocks the binding of C3bi-coated erythrocytes to monocytes. Thus, the C3bi molecule may be the ligand for HMac-1. A similar ligand for HLFA may be present in lymphocyte membranes; at present, however, no such ligand has been found. Springer et al. (10) have pointed out that both adhesion of CTL to target cells and adhesion through CR₃ occur through an Mg²⁺-dependent step. These interactions were blocked by HLFA and HMac-1, respectively. The mechanism by which lymphocytes and macrophages interact with other cells are possibly similar.

The HLFA, HMac-1, and p150-93 molecules appear to constitute a family of leukocyte antigens analogous to MHC antigens, immunoglobulins, and the hemoglobins in which subunits are shared. In analogy to other protein families with shared subunits, homology at the amino acid level may also be found for the α -subunits of HLFA and HMac-1. The structural and functional homology between these antigens suggests that they are possibly products of closely linked homologous genes.

Acknowledgments. We thank Dr. A. J. McMichael for the generous gift of hybridoma lines, and Dr. Mette Strand for a critical reading of the manuscript. We are also grateful to Dr. James Burdick for the gift of tissue samples.

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